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Article Addendum

The mechanism of action of PA-824

Novel insights from transcriptional profiling

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The bicyclic nitroimidazole PA-824 is a pro-drug with a very complex mechanism of action active against both replicating and hypoxic, non-replicating *Mycobacterium tuberculosis*. Microarray analysis of the mode of action of PA-824 showed a puzzling mixed effect both on genes responsive to both cell wall inhibition (like isoniazid) and respiratory poisoning (like cyanide). The aerobic killing mechanism of this drug appears to involve inhibition of cell wall mycolic acid biosynthesis through an as yet unknown molecular mechanism. However, the structure-activity relationships governing aerobic activity do not parallel the relationships determining anaerobic activity. Based on the metabolite profiling of PA-824 and various derivatives by Ddn-mediated activation, we have shown that PA-824 acts directly as an NO donor.[1] This respiratory poisoning through nitric oxide release seemed to be a crucial element of anaerobic activity by PA-824. The effect of PA-824 on the respiratory complex under hypoxic non-replicating conditions was also manifested in a rapid drop in intracellular ATP levels, again similar to that observed by cyanide treatment. Thus, transcriptional profiling provided valuable clues to elucidating the molecular mechanism of mycobacterial killing.

Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis (TB), has an amazing ability to persist in the human host.[2] The current TB drugs are highly effective against actively replicating bacilli and largely ineffective against persistent forms, leading to a current interest in developing new TB drugs which target persistent bacilli.[3] Bicyclic nitroimidazoles like PA-824 and OPC-67683 are an interesting class of anti-tuberculosis compounds that have inhibitory activity against both actively replicating and hypoxic non-replicating Mtb.[4,5] Both compounds are pro-drugs activated by a deazaflavin (cofactor F_{420}) dependent nitroreducase (Ddn).[6,7] Treatment of aerobically-replicating cells with PA-824 rapidly disrupts the formation of ketomycolates with concomitant accumulation of hydroxymycolates,[8] a class of mycolic acids that are major constituents of the cell envelope of Mtb. OPC-67683, a related bicyclic nitroimidazole, was optimized chemically based upon its ability to inhibit mycolic acid synthesis.[7] However, while there is obviously a connection with cell-wall inhibition under aerobic conditions, this effect seemed unlikely to be responsible for cell killing under non-replicating conditions since the bacilli do not extensively remodel mycolic acids under anaerobiosis.[9] Further, no other known mycolic acid synthesis inhibitors kill Mtb cells under hypoxic non-replicating conditions.

To gain further insight into the mechanism of action, we examined the transcriptional profiles of Mtb treated with PA-824 under aerobic conditions and compared them with transcriptional profiles of 430 inhibitors of mycobacterial metabolism.[10] These studies revealed that PA-824 had a complex mode of action with significant effects on transcription of genes responsive to known inhibitors of cell wall synthesis (such as isoniazid, thiolactomycin, ethionamide and cerulenin) as well as on genes responsive to respiratory poisons (such as potassium cyanide) (Fig. 1). Unfortunately, such transcriptional profiling can not be done with hypoxic cells because they have extremely low basal transcription rates. Ultimately this data suggested that the drug has two discrete targets, one from each class. To confirm this, expression of a few highly responsive genes with PA-824 treatment under aerobic conditions was confirmed by the quantitative reverse transcription-PCR analysis (Fig. 2). PA-824 treatment resulted in an upregulation of the *fasI* gene as well as many genes in the *fasII* operon; along with *efpA* and *iniBAC* operon. Several cell wall biosynthesis inhibitors have been shown to upregulate *iniBAC* operon.[10,11]

Unlike other mycolic acid synthesis inhibitors, PA-824 treatment also resulted in an upregulation of the *cyd* operon (*cydA*, *cydB*, *cydD* and *cydC*) encoding the non-proton-pumping cytochrome *bd* oxidase, the nitrate reductase *narGHIJ* and other genes involved in respiration. This transcriptional profile was very similar to cytochrome *c* oxidase-specific inhibitors like potassium cyanide (Fig. 1). Respiratory inhibitors, such as potassium cyanide, rapidly change the redox status of the cells, an effect that can be measured by examining the quinol/quinone pool. Like cyanide, PA-824 dramatically shifted

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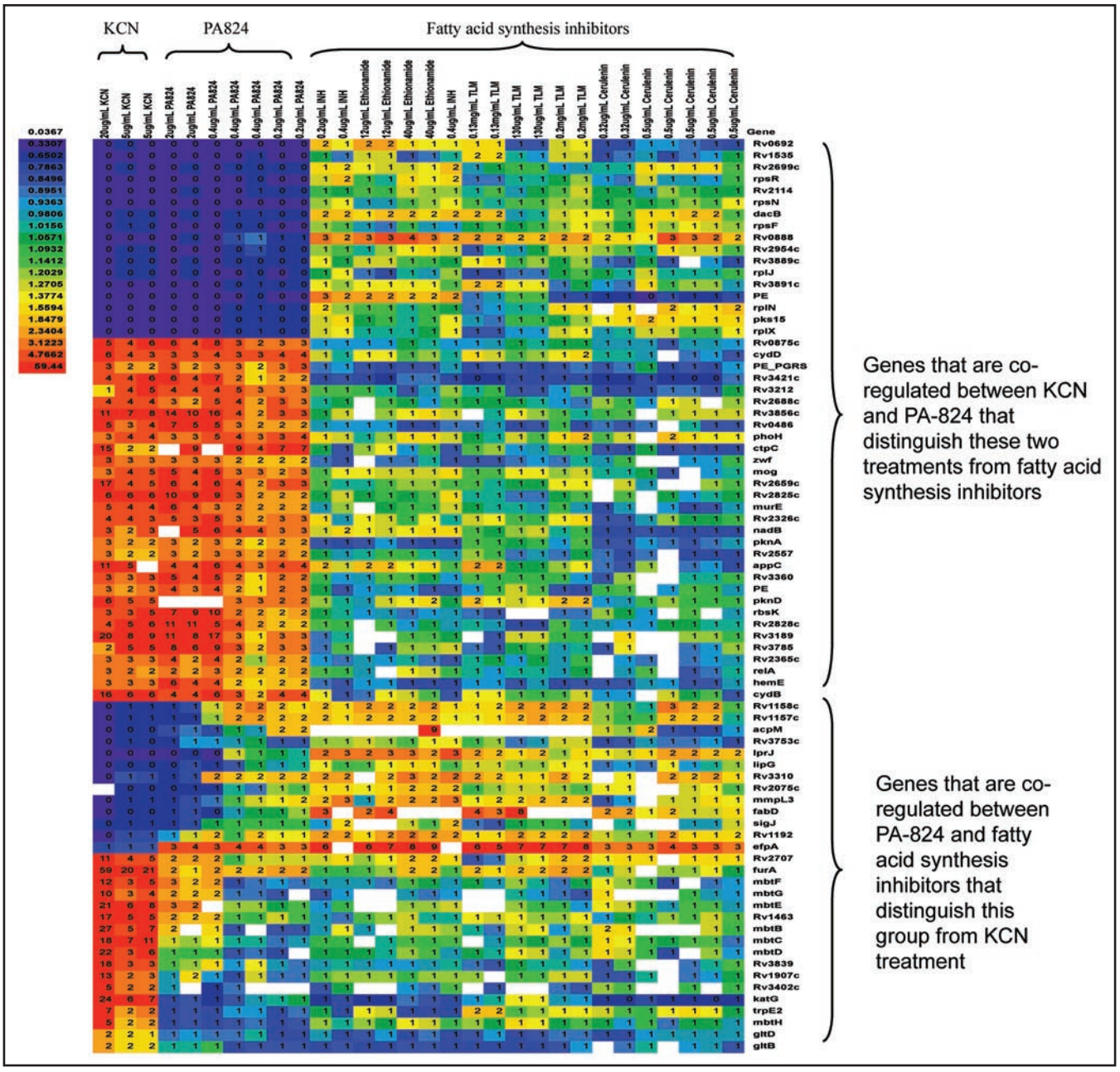

Figure 1. Transcriptional response profiles of *M. tuberculosis* to PA-824 and known respiratory and fatty acid synthesis inhibitors. Heat map rendered table of gene expression changes for those genes that predictively associate PA-824 with either KCN or fatty acid synthesis inhibitors as described earlier.[10] Inset shows color scale for expression ratios.

the predominant isoprenoid quinol/quinone ratio ($MK9H_2$/MK9) in a time and concentration dependent manner (Fig. 3). The effect of PA-824 on the respiratory complex under hypoxic non-replicating conditions was also manifested by a rapid drop in intracellular ATP levels (Fig. 4). This drop occurred within 2 hours of compound addition, whereas aerobically replicating cells treated with PA-824 did not show a drop in ATP within the first 24 hours of treatment (results not shown).

These observations led us to believe that the mechanism of action of PA-824 involved the inhibition of a fundamental component of energy metabolism and also encouraged us to look into the mechanism of Ddn-mediated bioreduction of these compounds in more detail. Based on metabolite profiling of PA-824 and its analogues by Ddn, we have recently shown that these bicyclic nitroimidazoles act as [NO] donors, and that NO release from various PA-824 derivatives correlated well with the anaerobic killing of Mtb.[1] Respiratory poisoning through nitric oxide release seems to be the crucial element of the anaerobic activity by PA-824. Thus PA-824 acts as a "suicide bomb" releasing toxic NO within mycobacterial cells and NO possibly reacts with cytochromes/cytochrome oxidase to interfere with the electron flow and ATP homeostasis under hypoxic non-replicating conditions. This NO-releasing effect of PA-824 is not sufficient to kill aerobically replicating cells. Likewise cyanide has little effect on aerobically replicating Mtb. Maintenance of an energised membrane and ATP homeostasis have been shown to be significant points of metabolic vulnerability in hypoxic non-replicating mycobacteria.[12,13]

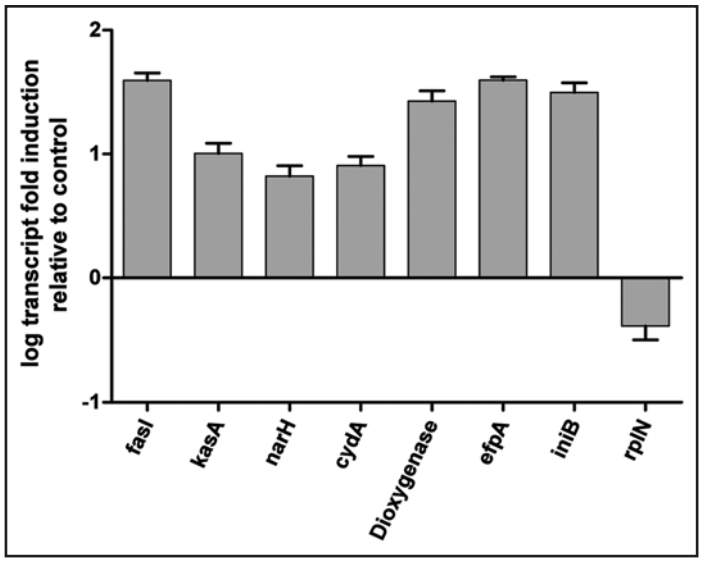

Figure 2. qRT-PCR analysis of selected genes with PA-824 treatment. Early mid-log phase cells treated with PA-824 2 μg/ml for 2 hrs prior to RNA extraction and qRT-PCR performed by Taqman analysis as described.[10] Expression levels of genes normalized to levels of the sigA mRNA.

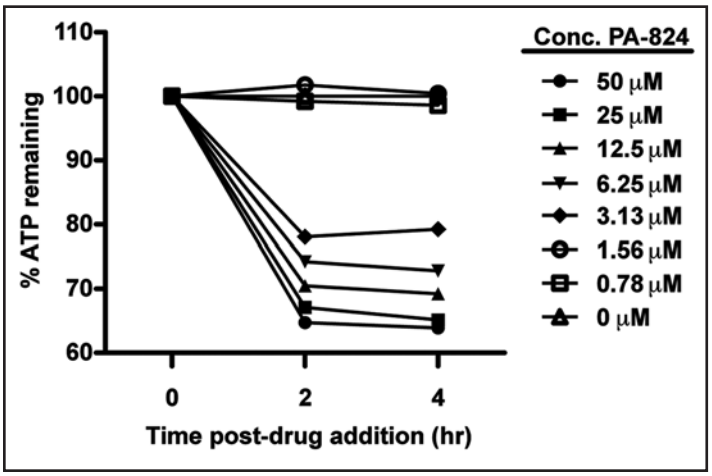

Figure 4. Effect of PA-824 treatment on cellular ATP levels on NRP-2 cells. Intracellular ATP was quantified by using the BacTiter-Glo Microbial Cell Viability Assay Kit (Promega). Anaerobically adapted cells were treated in white 96-well plates (100 μl/well) with different concentrations of PA-824 or DMSO alone at a final DMSO concentration of 0.2%. At 0, 2 and 4 hours, plates were removed from the anaerobic chamber and 20 μl of BacTiter-Glo reagent to each well. Luminescence was recorded 10 min after incubation in a Fluostar Optima plate reader (BMG Labtech).

In Mycobacterium, NO is known to have multiple targets including DNA,[14] as many as 29 mycobacterial enzymes,[15] including the ATP synthase, Pks13, RpoB and recently has been shown to displace copper in a toxic form from a metallothionein.[16] However, under aerobic conditions even though NO is released in mycobacterial cells,[1] it doesn't seem to play a critical role in cell killing. It is likely because the inhibition of cytochrome *c* oxidase by NO is reversible in the presence of oxygen.[17,18]

Thus, transcriptional profiling of PA-824 provided a fundamental insight into the involvement of respiratory poisoning and

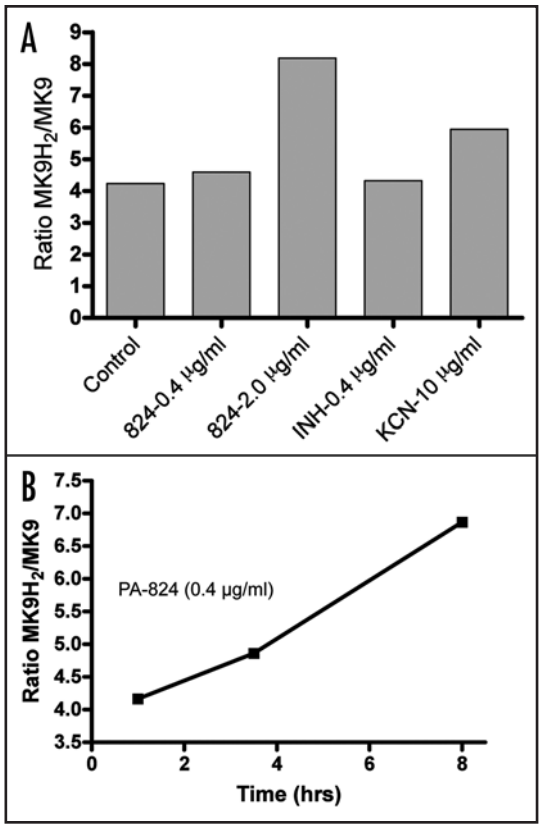

Figure 3. Effect of PA-824 on the redox status of menaquinol/menaquinone ($MK9H_2$/MK9). Early mid-log phase cells were treated with PA-824 for 1 hr (A) or as indicated (B) before cells were pelletted for menaquinone analysis as described.[10] Shown is the typical result of one of two independent experiments.

was a vital clue to elucidating the anaerobic mechanism of action. Of course, predictions based on transcriptional profiling need supporting evidence and often they merely generate hypotheses for further work. In the case of PA-824, the microarray data primed us to consider chemically plausible ways to generate a non-specific toxic metabolite.

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